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STUDY OF SINAPIC ACID, CAFFEIC ACID, VANILLIC ACID AND BOUGAINVILLEA EXTRACTS: EVALUATING THEIR EFFECT ON PROLIFERATION OF CHICK EMBRYO FIBROBLAST CELLS AND DETERMINING THEIR ABILITY TO REDUCE MTT REAGENT

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ABSTRACT

Plant polyphenols exhibit antioxidant and anticancer activities, and their cytotoxic effects are evaluated by MTT assay. Some polyphenols and plant extracts have potential to reduce the MTT reagent, thereby influencing experimental outcomes. This study aims to evaluate the effects of sinapic acid (SA), caffeic acid (CA), vanillic acid (VA) and bougainvillea extracts (BEE yellow flower[YF] and BEE white flower [WF]), on the growth of chick embryo fibroblast cells (CEF cells), and to investigate their interaction with the MTT reagent. The MTT readings of SA and CA treated cells increased with increasing concentration, suggesting cell growth, whereas for BEE (YF) and BEE (WF), readings decreased, indicating growth inhibition. However, the morphology of CA and BEE (WF) treated cells appeared rounded, indicating potential cytotoxic effects. In contrast, cells treated with SA, VA, and BEE (YF) did not show any noticeable changes in morphology. Cell-free MTT assays were performed to investigate the interaction of polyphenols and plant extracts with the MTT reagent. The MTT absorbance readings for SA and CA exhibited a dose-dependent increase, suggesting their capacity to effectively reduce the MTT reagent compared to VA. Both bougainvillea extracts also demonstrated the capacity to reduce the MTT reagent in a dose-dependent manner in the cell-free system. The MTT reduction potential of polyphenols followed the order CA > SA > VA, while for bougainvillea extracts, it was BEE (WF) > BEE (YF). The IC50 values were 21.15 µM for SA, 17.3 µM for CA, >1000 µM for VA, 46.03 µg/ml for BEE (YF) and 21.08 µg/ml for BEE (WF).

Keywords: *Sinapic acid, Caffeic acid, Vanillic acid, Bougainvillea ethanolic extracts, MTT assay*

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INTRODUCTION

Plant polyphenols are organic compounds typically characterised by two or more phenol units in their structure, and they are categorised into phenolic acids, flavonoids, anthocyanins and tannins (Asensi *et al*. 2011). Polyphenols offer benefits that include defense against oxidative stress, diabetes, cardiovascular disease, neurodegenerative disease and aging (Niedzwiecki *et al*. 2016). Plant polyphenols are further classified into cinnamic acids and benzoic acids, and they exist in free form or conjugated with ethers, esters and other molecules (Anantharaju *et al*. 2016); (Bonta 2020); (Srinivasulu *et al*. 2018); (Rahman, Costa de Camargo and Shahidi 2018). Anantharaju *et al*. (2016) noted that the structural features essential for the antitumor activity of phenolic compounds encompass aromatic rings, unsaturated substituted chains and the location and quantity of free hydroxyl groups.

Dietary polyphenols such as sinapic acid (SA) (3,5-dimethoxy-4-hydroxycinnamic acid) and caffeic acid (CA) (3,4-dihydroxycinnamic acid) are widely found in various fruits, tea, coffee, vegetables, cereal grains, oilseed crops, certain spices and medicinal plants (Martinović and Abramović 2014); (Chen 2016); (Jiang *et al*. 2005); (Bryngelsson, Dimberg and Kamal-Eldin 2002); (Castellari *et al.* 2002). Vanillic acid (VA) (4-hydroxy-3-methoxybenzoic acid) is present in various sources including the Chinese medicinal plant *Angelica sinensis*, benzoin, soybeans, olives (Dandekar and Wasewar 2020); (Duke 1992), as well as in fruits, sherry, rice grains, brandy, bourbon, scotch, Canadian whisky and red and white wines. The antioxidant properties of these dietary polyphenols can be utilised to assess their potential anti-cancer activity and cytotoxic effects.

Bougainvillea, a well-known ornamental plant, exhibits varied polyphenol compositions across its different parts such as leaves, branches, flowers and flowers bracts. These polyphenols are noted for their distinct pharmacological activities (Kaisoon, Konczak and Siriamornpun 2012); (Abarca-Vargas, Malacara and Petricevich 2016); (Soares *et al*. 2017). SA, CA and VA are identified in bougainvillea flower and leaf extracts (Kaisoon, Konczak and Siriamornpun 2012). Pharmacological investigations of these extracts and isolated compounds have demonstrated various activities including analgesic (Jawla, Kumar and Khan 2011); (Bhat *et al*. 2011), antidiabetic (Jawla, Kumar and Khan 2011), immunomodulatory (Figueroa *et al*. 2015), antioxidant (Islam *et al*. 2016; Figueroa *et al*. 2014; Medpilwar *et al*. 2015) and cytotoxic effects (Kaisoon, Konczak and Siriamornpun, 2012; Lamien-Meda *et al*. 2008).

Measuring cell viability in vitro in response to isolated chemical compounds or crude plant extracts using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cytotoxicity assay is a widely accepted and reliable method. The principle of the assay involves the reduction of MTT by mitochondrial dehydrogenases present in live cells. This reduction produces formazan crystals, which are solubilised with a suitable solvent to form a purple-coloured solution that can be measured spectrophotometrically. The intensity of the purple colour correlates with cellular metabolic activity. The intensity of the purple colour is directly proportional to cellular metabolic activity. However, studies by (Talorete *et al*. 2006) demonstrated that flavonoids such as quercetin, rutin and luteolin can reduce MTT even in the absence of live cells. They found that Dulbecco's Modified Eagle's Medium (DMEM) showed significant MTT reduction compared to RPMI 1640 and F12 media, and that increasing serum concentration led to larger formazan crystal sizes. Additionally, Bruggisser *et al*. (2002) and Wisman *et al.* (2008) reported direct reduction of MTT to formazan by certain phenolics like kaempferol and epigallocatechin gallate (EGCG) in cell-free conditions. Certain plant extracts and flavonoids including quercetin, EGCG, rutin, resveratrol, and tea polyphenols have also been shown to interfere with the MTT assay (Wang, Henning and Heber 2010); (Somayaji and Shastry 2021).

In this study, chick embryo fibroblast cells (CEF cells) were employed as a model system to investigate the impact of polyphenols such as SA, CA, VA and Bougainvillea plant extracts (BEE yellow flower [YF] and BEE white flower [WF]) using the MTT cytotoxicity assay. The growth inhibitory and cytotoxic effects of these compounds were evaluated based on morphological changes in the cells and MTT assay results. Additionally, experiments were conducted in a cell-free system to assess the interaction of these polyphenols and plant extracts with the MTT reagent.

METHODS

Chemicals and Reagents

CA and VA (95% purity) were purchased from PROCHEMDEV Pvt LTD, SA, MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals (St. Louis, MO). All other chemicals used were of analytical reagent (AR) grade.

Preparation of Polyphenol Samples

SA, CA and VA were dissolved in 100% DMSO to prepare 1 M stock solutions, which were subsequently filtered using a 0.45 µm syringe filter. Filtered aliquots of these stocks were stored at –20°C and diluted as needed in sterile DMSO to prepare working stocks ranging from 10 mM–1,000 mM. These working stocks were further diluted in complete DMEM (with 10% FBS) to achieve final concentrations ranging from 10 µM–1,000 µM for cytotoxicity assays.

Preparation of Leaf Extracts

Two varieties of Bougainvillea plants with YF and WF blooms were selected for this study. Fresh leaves were thoroughly washed with distilled water and then dried in an oven at 40°C. The dried leaves were crushed and subjected to ethanolic extraction using a soxhlet apparatus with 15 cycles of extraction. The resulting ethanolic extract was concentrated using a rotary evaporator, and the final residue was weighed and aliquoted into 20 mg portions in sterile eppendorf tubes, which were then stored at 4°C. Each aliquot was reconstituted in 1% DMSO, filter sterilised, suitably diluted in growth medium and utilised for cytotoxicity assays.

Chick Embryo Fibroblast Culture

Seven-day-old fertilised chicken eggs were obtained from the Central Poultry Development Organization, Goregaon, Mumbai. The chick embryo was dissected according to a standard protocol (Goldman 2006), and fibroblast cells were cultured in growth medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS, v/v), 0.5% antibiotic mix and 0.5% gentamicin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. Culture flasks containing cells with optimal density (70% confluency) and healthy morphology were trypsinised and used for the MTT assay.

MTT Cytotoxicity Assay in CEF Cells

The MTT cytotoxicity assay of SA, CA, VA, BEE (YF) and BEE (WF) was studied by MTT assay (Medpilwar *et al*. 2020) with slight modifications. CEF cells were plated at the density of 4,000 cells per well in flat bottom 96-well plate and incubated at 37°C in a humidified incubator containing 5% CO_2. After 24 hours of seeding, cells were treated with 5 µM–500 µM concentration of SA, CA and VA and 10 µg/mL–500 µg/mL of BEE (YF) and BEE (WF) extracts diluted in growth medium. Cells treated with growth medium containing 0.1% DMSO were used as a vehicle control (VC) for polyphenol-treated cells, while cells treated with 0.5% DMSO served as a VC for ethanolic extract-treated cells. Wells (without cells) containing only growth medium served as media blank. After 24 hours of treatment, cell morphology of treated and untreated CEF cells was carefully observed under an inverted microscope. For MTT assay, most of the media was aspirated from each well followed by addition of 40 µl MTT solution (5 mg/mL in 1 × PBS). After 4 hours, formazan crystals formed were dissolved by adding 100 µl of 100% DMSO per well and subjecting the plates to gentle agitation on a plate shaker for 30 minutes. Spectrophotometric absorbance at 540 nm was determined on an ELISA plate reader (Byonoy, Germany). All the experiments were performed in triplicates.

MTT Cytotoxicity Assay in Cell-free System

The MTT cytotoxicity assay was conducted in a cell-free system to investigate potential chemical interactions between SA, CA, VA, BEE (YF) and BEE (WF) and the MTT reagent. All the three polyphenols and BEE extracts were diluted in growth medium to obtain respective concentrations and 100 µl of each concentrations were added per well. After 24 hours, 90 µl of media was aspirated from each well and MTT solution was added. After 4 hours of incubation, the formazon crystals formed were inspected under inverted microscope and then dissolved by adding DMSO, absorbance was measured on an ELISA plate reader at 540 nm. All the experiments were performed in triplicates.

Statistical Analysis

Graphpad Prism (version 9.4.0) software was used for all statistical analysis. The experimental data were expressed as mean ± SD. Statistical significance between the polyphenol treated (cell free system) and untreated (media control) groups was evaluated by one-way ANOVA with Tukey's post hoc test, p-value set as ($p < 0.05$). Mann Whitney U test was employed to evaluate statistical significance between the groups comprising of normalised absorbance and non-normalised absorbance. The normalised absorbance was used to calculate percent relative cell viability using formula (Absorbance of treated) / (Absorbance of vehicle control) × 100. The IC50 values were calculated from linear regression analysis.

RESULTS

MTT Assay of CEF Cells Treated with SA, CA and VA

CEF cells were exposed to SA, CA and VA at concentrations ranging from 25 µM–500 µM for 24 hours. The absorbance values in the MTT assay for sinapic acid and caffeic

acid increased dose-dependently, indicating no inhibitory effect on CEF cells. In contrast, treatment with VA resulted in reduced absorbance values compared to the control, suggesting an inhibitory effect of VA on CEF cells. These absorbance values remained consistent across all concentrations of VA tested.

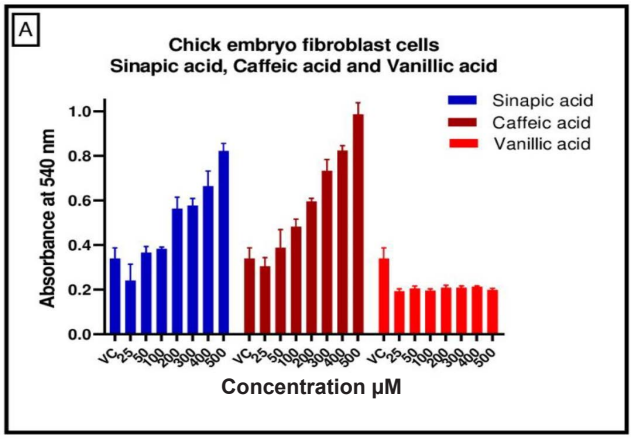

Figure 1A: MTT assay for CEF cells treated with SA, CA and VA.

The absorbance values for cells treated with SA and CA increased in a dose-dependent manner as compared to control suggesting no inhibitory effect against CEF cells. For CEF cells treated with VA, the absorbance values reduced as compared to control, indicative of inhibitory effect of VA at all the concentrations studied. Data is a representative of triplicates, mean ± SE.

MTT Assay of CEF Cells Treated with BEE (YF) and BEE (WF) Extracts

CEF cells were exposed to ethanolic extracts of bougainvillea at concentrations ranging from 6.25 µg/mL–50 µg/mL. When treated with BEE (YF) extracts, the absorbance values of the cells were comparable to the control, indicating no inhibitory effect up to a concentration of 50 µg/mL. In contrast, CEF cells treated with BEE (WF) extracts showed a relative decrease in absorbance values compared to the control. At a concentration of 50 µg/mL, there was a 1.8-fold reduction in absorbance values, indicating an inhibitory effect against CEF cells.

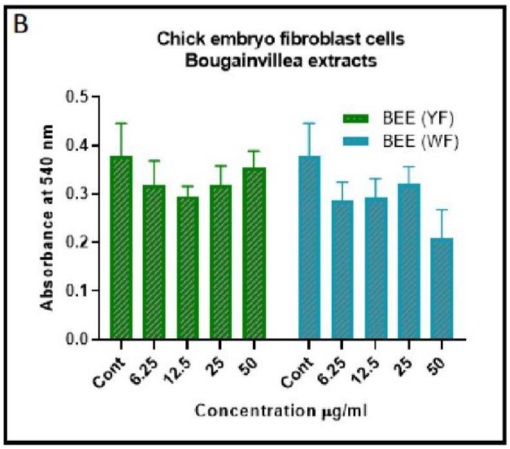

Figure 1B: MTT assay for CEF cells treated with BEE (YF) and (WF).

The absorbance values for CEF cells treated with BEE (YF) extracts were comparable to control, indicating no inhibitory effect. For CEF cells treated with BEE (WF), a relative decrease in the absorbance values seen with increasing concentration suggesting an inhibitory effect. Data shown is a representative of triplicates, mean ± SE.

Morphology of Chick Embryo Fibroblast Cells Treated with SA, CA and VA

The morphology of untreated control cells, vehicle control (DMSO-treated cells) and polyphenol-treated CEF cells was examined using an inverted microscope. The morphology and cell density of cells treated with 0.1% DMSO (VC) were comparable to the control cells. CEF cells were treated with SA at concentrations ranging from 5 µM–1,500 µM. At concentrations up to 50 µM, there was minimal cell density and no significant morphological alterations were observed, indicating no inhibitory effect. However, at concentrations ranging from 100 µM–300 µM, there was a significant increase in cell density compared to the control, suggesting that SA promotes cell proliferation at these concentrations. At 500 µM, 800 µM, 1,000 µM and 1,500 µM dose, the cell density decreased relatively and a negligible population of rounded cells was evident with increasing concentration of SA, indicating an inhibitory effect. Thus sinapic acid exhibits inhibitory action against CEF cells at > 500 µM concentration.

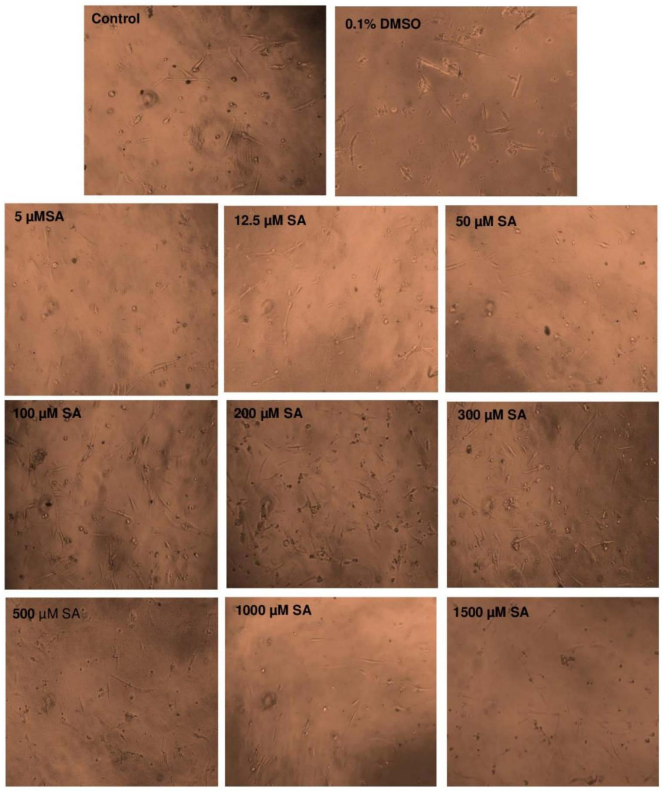

Figure 2: Morphology of CEF cells treated with SA after 24 hours treatment.

SA did not induce any significant morphological alterations in CEF cells up to a concentration of 1,500 µM, however at concentrations > 500 µM, the cell density decreased in dose-dependent manner indicating an inhibitory effect against CEF cells, magnification 20×.

CA at concentration of 5 µM–25 µM did not induce any morphological alterations of CEF cells, however at concentrations from 50 µM–200 µM, the population of CEF cells with flat morphology became scarce and numerous cells of rounded off morphology were evident, suggesting the cytotoxic effects of CA. The presence of significant population of rounded cells at concentrations > 200 µM, indicates CA exhibits cytotoxicity against CEF cells.

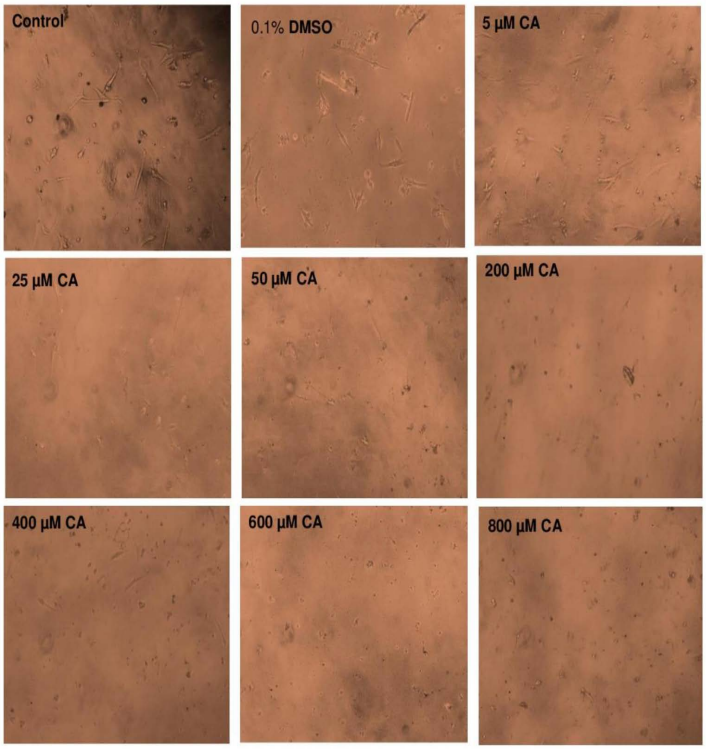

Figure 3: Morphology of CEF cells treated with CA after 24 hours treatment.

Treatment with CA induced morphological alterations in CEF cells in dose-dependent manner indicating cell cytotoxicity against CEF cells. Magnification 20×.

Effect of VA was studied at concentrations from 10 µM–1,000 µM. Even at 1,000 µM concentrations, VA did not show any impact on cell density or exhibit any morphological alterations in CEF cells suggesting least inhibitory effect against CEF cells (see Figure 4). Thus CA showed the greatest ability to induce morphological alterations in CEF cells followed by SA and VA.

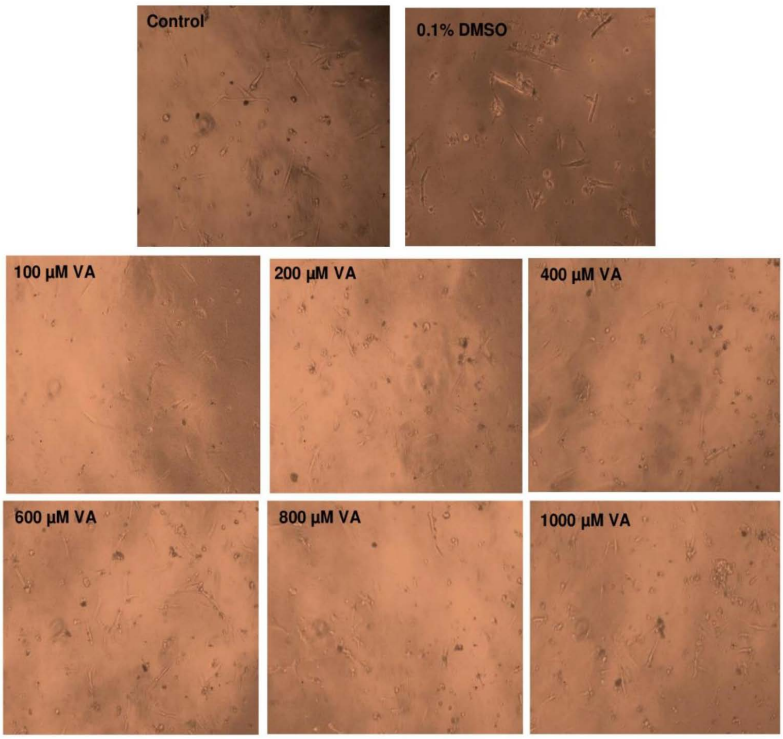

Figure 4: Morphology of CEF cells treated with VA after 24 hours treatment.

VA did not exhibit morphological alterations in CEF cells even up to a concentration of 1,000 µM. Magnification 20×.

Since the MTT assay measures cell viability, where absorbance correlates with the viable cell population, comparisons were made with morphological observations. Interestingly, despite CA inducing cytotoxicity in CEF cells, the MTT absorbance values increased with higher concentrations (see Figure 1A). SA also displayed inhibitory effects on CEF cells, yet the MTT readings increased in a dose-dependent manner (see Figure 1A). In contrast, VA did not inhibit cell growth, yet its MTT readings were lower compared to the control (see Figure 1A). These findings suggest a discrepancy between the morphological observations of polyphenol-treated cells and their MTT absorbance readings.

Morphology of Chick Embryo Fibroblast Cells Treated with BEE (YF) and BEE (WF)

The morphology of 0.5% DMSO-treated cells (VC) was comparable to control cells. For CEF cells treated with BEE (YF) extracts up to a dose of 50 µg/mL, no morphological alterations were observed. Even at concentrations of 75 µg/mL and 100 µg/mL, minimal morphological changes were observed, suggesting no cytotoxic effect (see Figure 5). On the contrary, BEE (WF) extract even at low concentration of 5 µg/mL, induced a significant alteration in the morphology of CEF cells. CEF cells treated with concentrations ranging from 15 µg/mL

to 100 µg/mL exhibited a rounded-off morphology, indicating significant toxicity to the cells (see Figure 6). Despite this pronounced toxicity observed with BEE (WF), the absorbance decreased by approximately 1.8-fold (see Figure 1B).

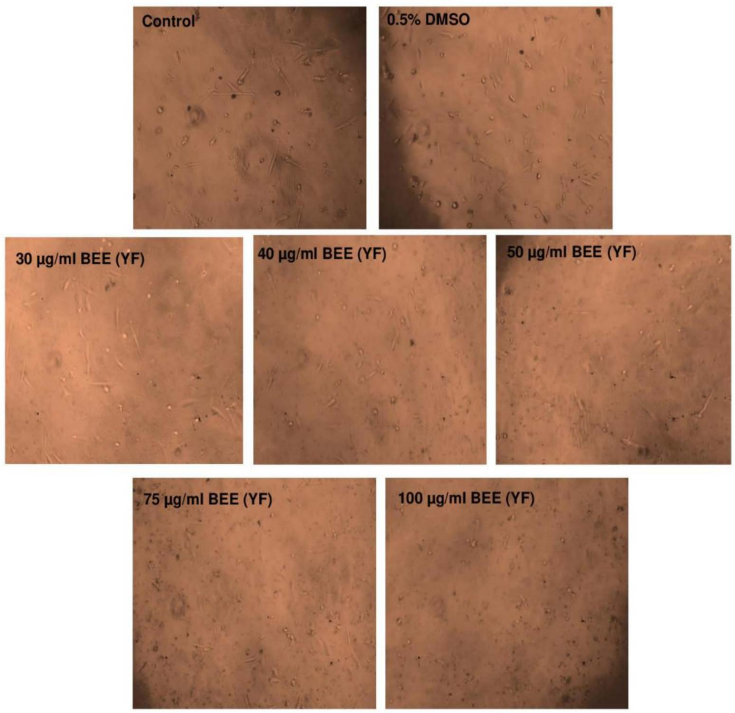

Figure 5: Morphology of CEF cells treated with BEE (YF).

BEE (YF) exhibited minimal morphological alteration in CEF cells at 20× magnification.

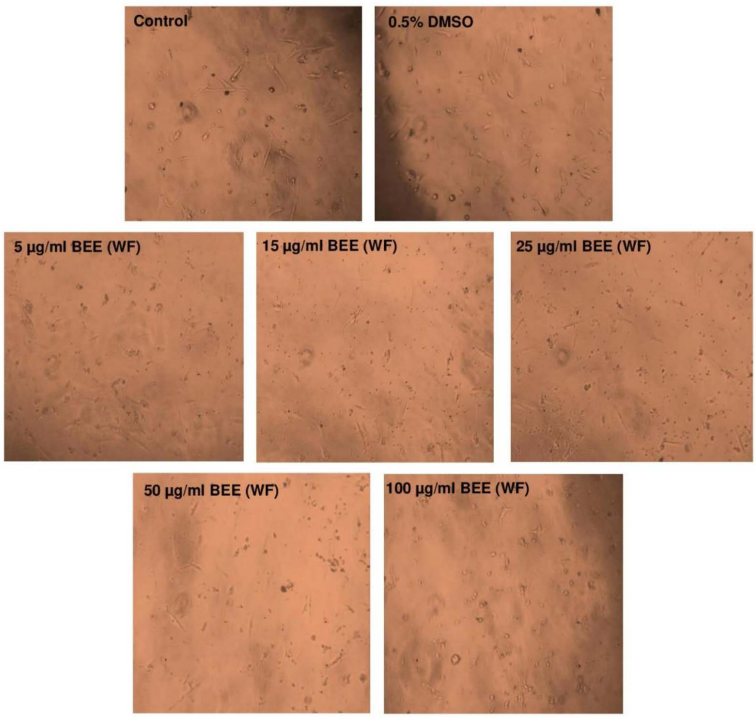

Figure 6: Morphology of CEF cells treated with BEE (WF).

BEE (WF) exhibited significant cell cytotoxicity against CEF cells as evident from rounded off cells at 20× magnification.

MTT Assay of SA, CA and VA and BEE (YF) and BEE (WF) in Cell-free System

The observed morphological changes in cells treated with polyphenols and plant extracts indicate that SA and BEE (YF) caused growth inhibition, while CA and BEE (WF) induced cytotoxic effects in CEF cells. However, the MTT readings were unexpectedly high, suggesting a possible interaction of these compounds with the MTT reagent.

To verify this, MTT assays were conducted in a cell-free system for all the polyphenols and BEE extracts. The absorbances of wells containing SA, CA and VA were compared with those of media control wells (see Figure 7A). The data underwent statistical analysis using one-way ANOVA followed by post-hoc Tukey's test. Tukey's multiple comparison test revealed a significant difference ($p < 0.0001$) in absorbance values between SA and CA compared to the media control (MC). However, the absorbance value of VA compared to MC was not significant ($p = 0.9869$). Tukey's comparison showed that the difference between SA and CA was not significant ($p = 0.0893$). However, the comparison of SA and CA versus VA was significant ($p < 0.0001$). Therefore, the potential of polyphenols to react with MTT was ranked as caffeic acid > sinapic acid > vanillic acid.

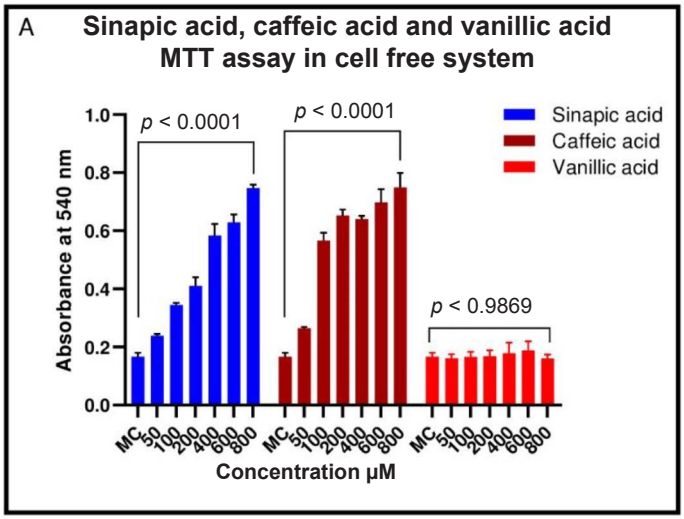

Figure 7A: MTT assay of SA, CA and VA in cell-free systems.

Both SA and CA showed a significant increase in absorbance values in correlation with dosage, indicating their capacity to reduce the MTT reagent. Data is a representative of triplicates, mean ± SE.

Tukey's multiple comparison test revealed a significant difference ($p < 0.0001$) in absorbance values between sinapic acid and caffeic acid compared to the media control (MC). However, the absorbance value of vanillic acid compared to MC was not significant ($p = 0.9869$).Top of In cell-free system, the absorbance values for both the plant extracts increased in a dose-dependent manner indicating their interaction with MTT reagent (see Figure 7B). The statistical analysis using one-way ANOVA with post-hoc Tukey's test indicates a significant difference ($p < 0.05$) in the absorbance values between BEE (YF) and BEE (WF) compared to the MC. The potential to reduce MTT was ranked as BEE (WF) > BEE (YF).

IC50 Value Determination of Polyphenols and Bougainvillea Extracts

For calculating inhibitory concentration 50 (IC50) values, the MTT readings of SA, CA, VA, BEE (YF) and BEE (WF)-treated cells was normalised by MTT readings of respective cell-free system controls. The graphs show the calculated IC50 values for SA, CA, VA, BEE (WF), and BEE (YF) (see Figure 8).

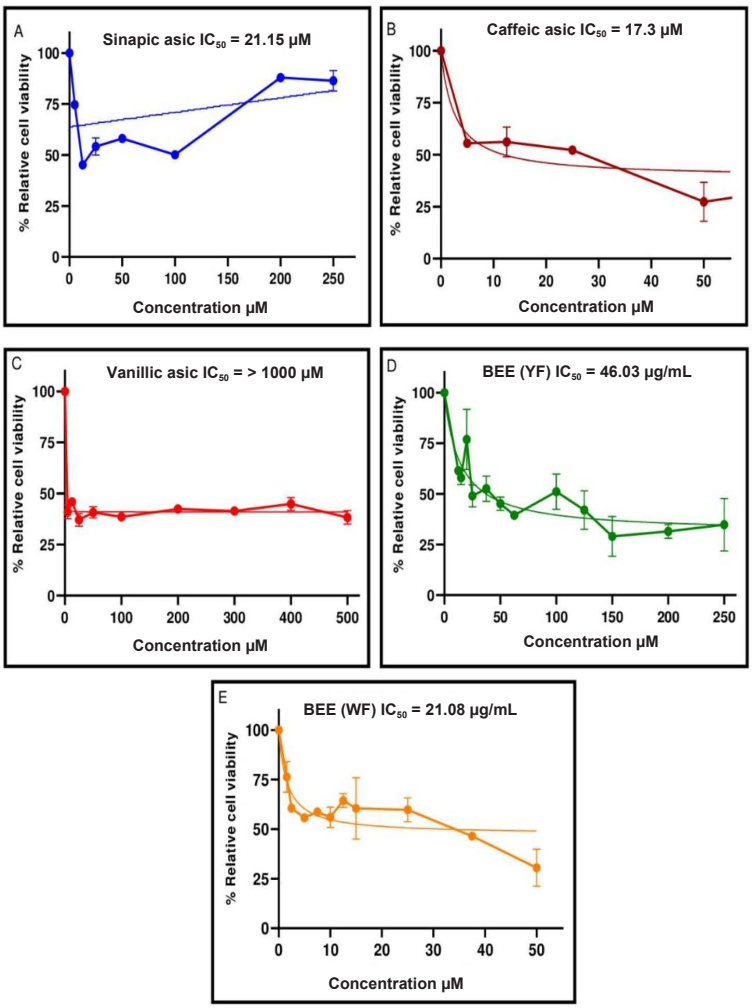

Figure 8: Percent relative viability of CEF cells treated with SA (A), CA (B), VA (C) and BEE (YF) (D) and BEE (WF) (E).

DISCUSSION

This study initially investigated the impact of SA, CA, VA, BEE (WF) and BEE (YF) on CEF cells through the MTT assay. We evaluated the growth inhibition and cytotoxic effects of these polyphenols and BEE on CEF cells by analysing MTT absorbance readings and monitoring changes in cell morphology. Our findings reveal that treatment with CA induced noticeable morphological changes in CEF cells, suggesting significant cytotoxic activity compared to SA and VA. BEE (WF) was also observed to cause morphological changes in CEF cells, unlike BEE (YF), suggesting cytotoxicity. However, the MTT absorbance

readings of cells treated with CA, SA and BEE as compared to control and DMSO-treated cells, surprisingly showed significantly higher absorbance values. This suggests that despite their growth inhibitory and cytotoxic effects on CEF cells, these compounds exhibit potential to react with MTT reagent. The MTT assay in a cell-free system clearly demonstrated that both CA and SA have the ability to reduce the MTT reagent. This was evidenced by observation of formazon crystals in a dose-dependent manner and an increase in MTT absorbance readings. VA showed minimal reaction with MTT reagent in the cell-free system. The order of MTT reduction ability was CA > SA > VA. Additionally, both plant extracts demonstrated dose-dependent reactions with MTT, indicating that a multitude of phytochemical constituents have potential to reduce MTT reagent.

Previous studies by (Talorete *et al.* 2006) have highlighted the ability of plant compounds and polyphenols to reduce the MTT reagent. Specifically, they reported that flavonoids such as quercetin, rutin, luteolin and apigenin can reduce MTT in the absence of cells, with this reduction potentially influenced by the type of growth medium and serum used. Flavonoids like EGCG, quercetin, resveratrol and rutin have been noted by (Somayaji and Shastry 2021) for their potential to reduce MTT to formazan, which can lead to misleading interpretations of results. Similarly, certain herbal extracts and antioxidants, including vitamin E and its isomers, have also demonstrated the ability to reduce MTT to formazan in cell-free systems, as reported by (Lim *et al.* 2015; Shoemaker, Cohen and Campbell 2004). Karakaş, Ari and Ulukaya (2017) observed that methanol extracts from *hypericum adenotrichum, salvia kronenburgii* and *pelargonium quercetorum* reacted with MTT, potentially yielding false-positive viability outcomes.

Therefore, while the MTT assay is commonly employed for assessing the cytotoxicity of natural products or crude plant extracts, it requires careful attention and vigilance during the assay to ensure accurate interpretation of results. Using highly concentrated plant extracts or excessively high concentrations of isolated compounds may potentially interact with the MTT reagent, leading to inaccurate results. Therefore, it is advisable to dilute the extracts appropriately to prevent any interference in the assay. The studies by Bruggisser *et al.* (2002) and Wisman *et al.* (2008) documented the direct reduction of MTT to formazan by phenolics, kaempferol and EGCG in cell-free conditions. They proposed washing cells to remove residual cytotoxic agents before adding MTT to minimise interference in the assay. However, this washing step may lead to cell loss. Therefore, we recommend normalising the absorbance values of polyphenols and plant extract-treated cells with those of cell-free systems when calculating percent viability. The calculated IC50 values for SA, CA and VA were 21.15 µM, 17.3 µM and > 1000 µM, respectively. For BEE (YF) and BEE (WF), the IC50 values were 46.03 µg/mL and 21.08 µg/mL, respectively.

We propose that the structure of SA, CA and VA may be correlated with their potential to reduce MTT (see Figure 9).

Figure 9: Structure of sinapic acid, caffeic acid and vanillic acid.

SA features a hydroxyl group at the C-4 position, with two methoxy groups at the C-3 and C-5 positions. CA, on the other hand, is hydroxylated at the C-3 and C-4 positions but does not have methoxy groups. VA is hydroxylated at the C-4 position, with a methoxy group at the C-3 position. The presence of phenolic hydroxyl and methoxy groups strongly influences the antioxidant properties of phenolic acids. Importantly, a higher number of hydroxyl groups generally corresponds to increased antioxidant activity. The phenolic hydroxyl group exhibits superior electron donating ability, thereby enhancing the antioxidant activity of other hydroxyl groups. This characteristic is also demonstrated by CA. The methoxy group is also recognised to influence the antioxidant properties of phenolic acids. In the cases of SA and VA, the presence of the methoxy group enhances their antioxidant activity. SA is substituted with two methoxy groups at the C-3 and C-5 positions, whereas VA has only one methoxy group at the C-3 position. According to (Chen *et al*. 2020), phenolic acids exhibit higher antioxidant activity when they contain a greater number of methoxy groups. Thus, it is concluded that SA exhibits stronger antioxidant properties compared to VA. The reduction of MTT to formazan by SA likely occurs through the transfer of a hydrogen atom from the phenolic group at the C-5 position (see Figure 10).

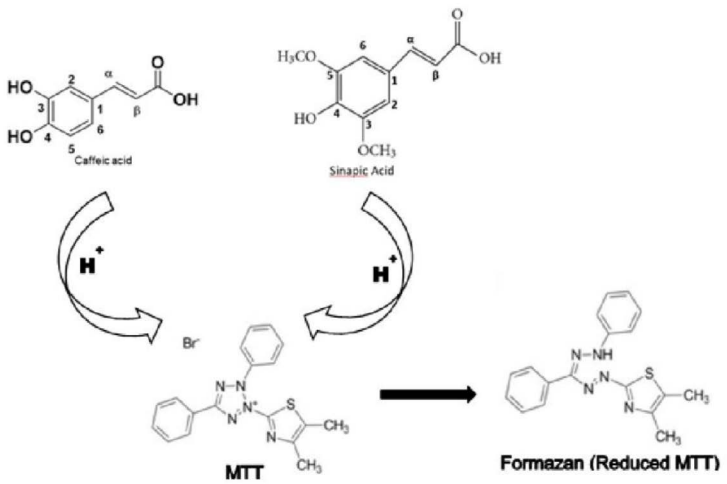

Figure 10: Transfer of hydrogen atom from SA and CA to MTT thereby reducing it to formazan.

In CA, the hydrogen atom is transferred from either of the hydroxyl groups at the C-3 or C-4 positions (see Figure 10). In contrast, VA has only one phenolic hydroxyl group, which results in a lower reduction potential compared to SA and CA. Consequently, VA shows less MTT reduction, as reported by (Talorete *et al.* 2006). Therefore, it is hypothesised that the reduction of MTT to formazan in the presence of phenolic acids (SA, CA and VA) occurs via hydrogen atom transfer. Importantly, the solvent polarity significantly influences the hydrogen atom transfer ability during MTT reduction, as noted by (Galano *et al.* 2011). The polar environment increases the reactivity of all three phenolic acids towards MTT.

The solvent polarity facilitates the interaction between cationic MTT and anionic phenolic acids (SA^-, CA^- and VA^-), forming binding complexes with MTT. This interaction can be corroborated by the observed increase in cell viability, as discussed by (Cheng *et al.*

2020). It is also important to note that the presence of phenolic hydroxyl (-OH) and methoxy (-OCH_3) groups lowers the ionisation potential values in SA and VA, thereby increasing their electron donation capacity and MTT reduction potential, as discussed by (Cheng *et al.* 2020).

CONCLUSION

The MTT assay is a widely accepted method for assessing cell viability and cytotoxicity of plant polyphenols and extracts. However, analysing and interpreting the data can be challenging. It is crucial to correlate MTT readings of treated and control groups with cell morphology during result interpretation. Careful selection of plant extract dilutions can reduce interference from plant pigments and particulate matter in the assay. Normalising the absorbance values of treated cells with those of respective cell-free systems can help mitigate potential errors in the results.

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